



Hutchison, S., Rae, C., Tesson, M., Babich, J., Boyd, M., and Mairs, R.  
(2014) Evaluation of melanin-targeted radiotherapy in combination with  
radiosensitizing drugs for the treatment of melanoma. *Cancer and Oncology  
Research*, 2(4), pp. 58-67.

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Version: Published

<http://eprints.gla.ac.uk/105768>

Deposited on: 05 May 2015

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# Evaluation of Melanin-Targeted Radiotherapy in Combination with Radiosensitizing Drugs for the Treatment of Melanoma

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**Abstract** The incidence of malignant melanoma is rising faster than that of any other cancer in the United States. An [<sup>131</sup>I]-labeled benzamide - [<sup>131</sup>I]MIP-1145 - selectively targets melanin, reduces melanoma tumor burden and increases survival in preclinical models. Our purpose was to determine the potential of radiosensitizers to enhance the anti-tumor efficacy of [<sup>131</sup>I]MIP-1145. Melanotic (A2058) and amelanotic (A375 and SK-N-BE(2c)) cells were treated with [<sup>131</sup>I]MIP-1145 as a single agent or in combination with drugs with radiosensitizing potential. Cellular uptake of [<sup>131</sup>I]MIP-1145 and toxicity were assessed in monolayer culture. The interaction between radiosensitizers and [<sup>131</sup>I]MIP-1145 was evaluated by combination index analysis in monolayer cultures and by delayed growth of multicellular tumor spheroids. [<sup>131</sup>I]MIP-1145 was taken up by and was toxic to melanotic cells but not amelanotic cells. Combination treatments comprising [<sup>131</sup>I]MIP-1145 with the topoisomerase inhibitor topotecan or the PARP-1 inhibitor AG014699 resulted in synergistic clonogenic cell kill and enhanced delay of the growth of spheroids derived from melanotic melanoma cells. The proteasome inhibitor bortezomib had no synergistic cytotoxic effect with [<sup>131</sup>I]MIP-1145 and failed to enhance the delay of spheroid growth. Following combination treatment of amelanotic cells, neither synergistic clonogenic cell kill nor enhanced growth delay of spheroids was observed.

**Keywords** Targeted Radiotherapy, Melanoma, Radiosensitizer

## 1. Introduction

According to the National Cancer Institute, the incidence of malignant melanoma has doubled in the last 30 years and continues to rise in the United States and UK. Chemotherapeutic agents have limited activity in the

treatment of metastatic melanoma and patients continue to have a poor prognosis. Response rates for metastatic melanoma with currently available treatments are poor and 10-year survival is less than 10% [1]. Thus, the advent of new, targeted therapies is expected to improve the poor results of current therapeutic regimens.

Targeted radiotherapy seeks to overcome the obstacles imposed by metastatic dissemination and the intolerance of normal tissue to ionizing radiation. Radiolabeled benzamide derivatives bind melanin and exhibit high uptake and retention in melanoma cells and in mice bearing melanoma tumors [2]. Alkylating benzamides have been shown to promote tumor growth delay in preclinical models [3] and high tumor uptake of radiolabelled benzamides has been observed *in vivo* [4]. Following the evaluation of a library of benzamide compounds, [<sup>131</sup>I]MIP-1145 (N-(2-diethylaminoethyl)-4-(4-fluoro-benzamido)-5-iodo-2-methoxy-benzamide) was selected for entry into clinical trials based on its significant melanin-specific tumor uptake and retention *in vitro* and *in vivo*, anti-tumor activity and radiochemical stability and solubility profile [5]. Our aim was to establish the most effective use of [<sup>131</sup>I]MIP-1145 treatment of melanoma by combination with radiosensitizing agents.

Several classes of drug have been proposed to enhance the anti-tumor potency of radiotherapy, including inhibitors of poly(ADP-ribose) polymerase-1 (PARP-1), topoisomerase and the proteasome. These agents were expected to increase radiation-induced DNA damage more effectively in rapidly proliferating cancer cells than normal melanocytes. The proteasome regulates the degradation of tumor suppressors, transcription factors and proteins involved in cell cycle control and high levels of proteasome activity have been observed in cancer cells [6]. Bortezomib is the first proteasome inhibitor approved for the treatment of multiple myeloma and has been reported to suppress the growth of solid tumors including melanoma [7]. Furthermore, bortezomib has been reported to radiosensitize tumors both

in experimental models [8] and in patients [9,10].

PARP-1 is a DNA repair enzyme which is over-expressed in some cancer cells [11]. PARP-1 inhibitors improve the efficiency and selectivity of DNA-damaging agents [12], indicating their potential as components of combination therapy. Ionizing radiation elicited greater damage to DNA in PARP-1-deficient mice and cell lines, and replicating tumor cells were sensitized to ionizing radiation by PARP-1 inhibitors [13]. Radiosensitization by PARP-1 inhibitors has been demonstrated in human and rodent cell lines [14] and in experimental tumors [12]. The PARP-1 inhibitor AG014699, a pro-drug of AG014447, has been shown to improve the efficacy of the DNA alkylating agent temozolomide in preclinical models of medulloblastoma [15] and neuroblastoma [16]. Furthermore, the radiosensitizing properties of AG14447 have also been demonstrated *in vitro* [17], suggesting similar potential for AG014699. AG014699 has been shown to be effective in clinical trials and to induce minimal toxicity in patients [18,19].

DNA unwinding during replication is regulated by topoisomerase. Drugs that target topoisomerase stabilize topoisomerase-DNA cleavable complexes, leading to irreversible DNA double-strand breaks and cell death. Combination therapy with topoisomerase poisons and targeted radiotherapy has great potential to improve treatment efficacy and decrease normal tissue toxicity [20]. In particular, the camptothecin analogue topotecan sensitizes tumors to radiation *in vitro* and *in vivo* [21-25]. Topotecan has also been shown to exhibit a tumor-selective radiation-sensitizing effect in clinical trials [26].

In this study we show that the topoisomerase poison topotecan or the PARP-1 inhibitor AG014699 synergized with [<sup>131</sup>I]MIP-1145 in the treatment of melanotic cells cultured as monolayers. Furthermore, these agents significantly enhanced the [<sup>131</sup>I]MIP-1145-induced delayed growth of melanotic multicellular tumor spheroids. In contrast, the proteasome inhibitor bortezomib failed to enhance the efficacy *in vitro* of treatment with [<sup>131</sup>I]MIP-1145.

## 2. Materials and Methods

### 2.1. Reagents

Topotecan was purchased from LKT Laboratories Inc (Minnesota, USA), AG014699 and bortezomib were purchased from Stratech Scientific Limited (Suffolk, UK). Stock solutions of agents were prepared in dimethyl sulfoxide (DMSO). The maximum DMSO concentration in culture medium was 0.1% (v/v). Radiolabeling of [<sup>131</sup>I]MIP-1145 was carried out as previously described [5].

### 2.2. Tissue Culture

Cell lines A375, A2058 and SK-N-BE(2c) were obtained from ATCC and were used in this study for less than 6

months after resuscitation. Cells were maintained in DMEM supplemented with 10% (v/v) (A375 and A2058 cells) or 15% (v/v) (SK-N-BE(2c) cells) fetal calf serum (Autogen Bioclear, Wiltshire, UK) and 1% (v/v) L-glutamine. All media were obtained from Gibco (Paisley, UK).

### 2.3. Cellular Uptake of [<sup>131</sup>I]MIP-1145

Cells were seeded at 10<sup>5</sup> per well in Costar 6-well culture plates and incubated for 2 to 3 days. Confluent cultures were incubated with 3.7 kBq [<sup>131</sup>I]MIP-1145 for various times up to 8 hours. At the indicated time, the medium was removed and cells were washed twice with PBS to remove the compound bound to the cell surface. Radioactivity was extracted by treatment with 10% (w/v) trichloroacetic acid for 1 h at 4°C. The activity in the extract was measured by beta-counting using a Wallac 1450 Trilux Microbeta liquid scintillation counter.

### 2.4. Clonogenic Survival Assay

Cells were seeded in 25 cm<sup>2</sup> flasks at 2.5 x 10<sup>5</sup> cells/flask. When cultures were in exponential growth phase, medium was removed and replaced with fresh medium containing [<sup>131</sup>I]MIP-1145, drugs or combinations of these agents. Three different combination treatment schedules were assessed: (i) [<sup>131</sup>I]MIP-1145 and drug administered simultaneously, (ii) [<sup>131</sup>I]MIP-1145 given 24 h before drug, (iii) [<sup>131</sup>I]MIP-1145 administered 24 h after drug. Cells were then incubated for 24 h at 37°C in 5% CO<sub>2</sub>. After treatment, cells were seeded for clonogenic survival assay as previously described [24,25]. Cells were incubated at 37°C in 5% CO<sub>2</sub> for 10 to 14 days. Colonies were fixed in methanol, stained with crystal violet solution (Sigma-Aldrich, UK) and counted.

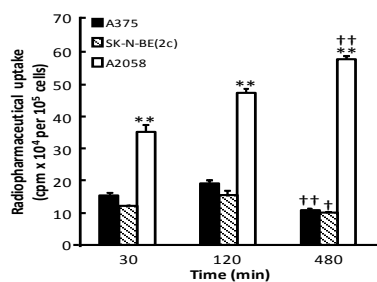
### 2.5. Combination Treatments

The cytotoxic interaction between [<sup>131</sup>I]MIP-1145 and drugs *in vitro* was assessed according to the method of Chou and Talalay [27], which is based on the median-effect principle. Briefly, clonogenic assay was carried out using a fixed dose ratio of drug to [<sup>131</sup>I]MIP-1145, based on the concentrations required to kill 50% of clonogens (IC<sub>50</sub>) of each single agent, so that the proportional contribution of each agent in the mixtures would be the same at all treatment intensities. The effectiveness of combinations was quantified by calculating a combination index (CI) at various levels of cytotoxicity. CI < 1, CI = 1 and CI > 1 indicate synergism, additivity and antagonism, respectively.

### 2.6. Multicellular Spheroids

SK-N-BE(2c) neuroblastoma cells were used as an amelanotic model because A375 cells did not form spheroids. Multicellular tumor spheroids (MTS) of A2058 or SK-N-BE(2c) cells were obtained using the liquid overlay

technique [28]. MTS were initiated by inoculating  $3 \times 10^6$  cells into a 25 cm<sup>2</sup> plastic flask coated with 1% (w/v) agar. After 3 to 4 days, aliquots of spheroids were transferred to sterile plastic 25 ml tubes and the spheroids were centrifuged at 12 x g for 3 min and re-suspended in 2 ml fresh culture medium containing drug. After treatment, the spheroids were washed twice by suspension in culture medium, followed by centrifugation at 12 x g. Spheroids of approximately 100  $\mu$ m in diameter were transferred individually into agar-coated wells of 24-well plates. Individual spheroid growth was monitored twice per week for at least three weeks using an inverted phase-contrast microscope connected to an image acquisition system. Two perpendicular diameters,  $d_{\max}$  and  $d_{\min}$ , were measured using image analysis software (ImageJ) and the volume,  $V$  ( $\mu$ m<sup>3</sup>), was calculated using the formula:  $V = \pi \times d_{\max} \times d_{\min}^2 / 6,000,000$  [28]. The area under the  $V/V_0$  versus time curve (AUC) was calculated for individual spheroids using trapezoidal approximation.



**Figure 1.** Uptake of [<sup>131</sup>I]MIP-1145 by A375, SK-N-BE(2c) or A2058 cells. Cells were incubated with 3.7 kBq/ml of [<sup>131</sup>I]MIP-1145 for various lengths of time, then cell-associated activity was determined by beta-counting. Data are means  $\pm$  SEM; n=3. \*\*  $P < 0.01$  compared to amelanotic cell lines, †  $P < 0.05$  and ††  $P < 0.01$  compared to 120 min.

## 2.7. Statistical Analysis

Data are presented as means  $\pm$  standard error of the mean (SEM), unless otherwise stated, with the number of independent repetitions provided in the legend to each figure. The uptake of [<sup>131</sup>I]MIP-1145 by melanoma cell lines was compared using Student's *t* test. A  $P$  value  $< 0.05$  was considered to be statistically significant and  $< 0.01$  highly significant. To test for differences in spheroid growth between experimental therapy groups the Kruskal-Wallis test was used with post hoc testing by the Mann-Whitney U

test with Bonferroni correction. Analysis was carried out using SPSS software.

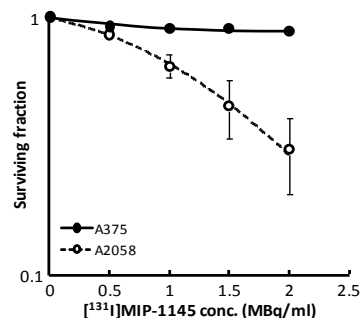
## 3. Results

### 3.1. Binding of [<sup>131</sup>I]MIP-1145 to Melanotic and Amelanotic Cells

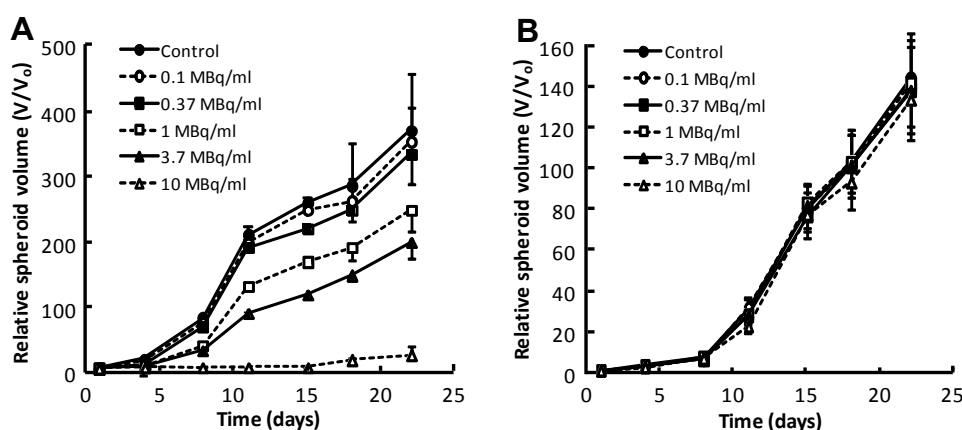
Binding of [<sup>131</sup>I]MIP-1145 to A375, SK-N-BE(2c) or A2058 cells is shown in Figure 1. Binding to melanotic cells (A2058) was significantly greater than to amelanotic cells (A375 or SK-N-BE(2c)) at all incubation times examined ( $P < 0.01$ ). [<sup>131</sup>I]MIP-1145 remained bound to melanotic cells for at least 8 hours after treatment with the radiopharmaceutical ( $P < 0.01$ ), whereas cell-associated radioactivity decreased in amelanotic cells 8 hours after treatment compared with earlier time points ( $P < 0.05$ ).

### 3.2. Selective Toxicity of [<sup>131</sup>I]MIP-1145 to Melanotic Cells

According to clonogenic assay, [<sup>131</sup>I]MIP-1145 was toxic to A2058 cells (melanotic), but not to A375 cells (amelanotic) (Figure 2). Furthermore, the growth of spheroids consisting of A2058 cells was delayed in a concentration-dependent manner by incubation with [<sup>131</sup>I]MIP-1145 at radioactivity concentrations of 0.1 to 10 MBq/ml (Figure 3A). In contrast, spheroids consisting of SK-N-BE(2c) cells were unaffected by treatment with [<sup>131</sup>I]MIP-1145 (Figure 3B).



**Figure 2.** Toxicity of [<sup>131</sup>I]MIP-1145. A2058 (melanotic) and A375 (amelanotic) cells were exposed to [<sup>131</sup>I]MIP-1145 for 2 hours, before cytotoxicity was assessed using clonogenic assay. Data are means  $\pm$  SEM, n=3..



**Figure 3.** The effect on the growth of multi-cellular spheroids of treatment with [ $^{131}\text{I}$ ]MIP-1145. Spheroids consisting of (A) A2058 cells or (B) SK-N-BE(2c) cells were exposed to [ $^{131}\text{I}$ ]MIP-1145 for 2 hours, and spheroid size was measured every 3 to 4 days. Data are expressed as mean spheroid volume at every time-point divided by original volume ( $V/V_0$ )  $\pm$  SEM of 24 spheroids per treatment.

**Table 1.** The effect of administration schedule of the combination of drug and [ $^{131}\text{I}$ ]MIP-1145 on the kill of clonogens derived from A2058 cells. Combination index values are means of 3 experiments for each alternative schedules of administration. CI values  $< 1$  indicate synergy and values  $> 1$  indicate partial antagonism. Administration schedules were: drug given 24 h before [ $^{131}\text{I}$ ]MIP-1145 (pre), simultaneous treatment with drug and [ $^{131}\text{I}$ ]MIP-1145 (sim.), drug given 24 h after [ $^{131}\text{I}$ ]MIP-1145 (post).

Drug	Schedule	25% kill	50% kill	75% kill
Topotecan	Pre	0.31	0.31	0.33
	Sim.	0.54	0.49	0.49
	Post	0.94	0.88	0.89
AG014699	Pre	0.84	0.72	0.62
	Sim.	0.66	0.57	0.49
	Post	1.64	1.36	1.13
Bortezomib	Pre	2.09	2.57	3.14
	Sim.	0.83	1.14	1.57
	Post	4.20	6.30	9.50

### 3.3. Topotecan and AG014699 Synergized with [ $^{131}\text{I}$ ]MIP-1145 in the Treatment of Melanotic Cells

Combination index analysis was performed to determine synergistic interaction between [ $^{131}\text{I}$ ]MIP-1145 and radiosensitizers. Agents were administered at fixed dose ratios, based on the IC<sub>50</sub> values of single agents. The following IC<sub>50</sub> values were observed following the treatment of A2058 cells with topotecan, AG014699 or bortezomib: 0.90 M, 7.88 M or 15.74 nM, respectively. In the case of A375 cells, the corresponding values were 2.39 M, 7.96 M or 9.83 nM respectively. Three different scheduling regimes were compared; (i) drug administered 24 hours before [ $^{131}\text{I}$ ]MIP-1145, (ii) drug and [ $^{131}\text{I}$ ]MIP-1145 administered simultaneously and (iii) [ $^{131}\text{I}$ ]MIP-1145 administered 24 hours after drug. In Table 1 are shown representative CI values calculated at a range of toxicity levels (25, 50 and 75% cell kill) for every administration

schedule. Topotecan, given in combination with [ $^{131}\text{I}$ ]MIP-1145, was synergistic (CI  $< 1$ ) regardless of dosing schedule. However, the administration of topotecan 24 hours before [ $^{131}\text{I}$ ]MIP-1145 or simultaneous administration of the two agents were more effective schedules than the administration of topotecan 24 hours after [ $^{131}\text{I}$ ]MIP-1145. Likewise, AG014699 enhanced the toxicity of [ $^{131}\text{I}$ ]MIP-1145 when it was administered 24 hours before [ $^{131}\text{I}$ ]MIP-1145 or when both agents were administered simultaneously. In contrast, the administration of AG014699 24 hours after [ $^{131}\text{I}$ ]MIP-1145 had an antagonistic effect (CI  $> 1$ ). Regardless of the sequence of administration of agents, combination treatments comprising bortezomib and [ $^{131}\text{I}$ ]MIP-1145 resulted in no synergistic interaction

**Table 2.** The effect of administration schedule of the combination of drug and [ $^{131}\text{I}$ ]MIP-1145 on the kill of clonogens derived from A375 cells.

Drug	Schedule	25% kill	50% kill	75% kill
Topotecan	Pre	1.16	1.59	2.20
	Sim.	0.95	1.15	1.41
	Post	1.02	1.28	1.60
AG014699	Pre	2.62	2.87	3.14
	Sim.	2.14	2.07	2.01
	Post	2.31	2.30	2.29
Bortezomib	Pre	2.93	2.62	2.35
	Sim.	2.83	2.18	1.68
	Post	3.48	2.55	1.88

### 3.4. [ $^{131}\text{I}$ ]MIP-1145 did not Synergize with Radiosensitizers in the Treatment of Amelanotic Cells

In contrast to the increased efficiency of clonogenic cell kill resulting from the treatment of melanotic A2058 cells with topotecan or AG014699 in combination with

[<sup>131</sup>I]MIP-1145, no synergistic interaction was observed following the treatment of amelanotic A375 cells with topotecan or AG014699 in combination with [<sup>131</sup>I]MIP-1145, regardless of the sequence of delivery of the agents (Table 2). Similarly, in the treatment of A375 cells with combinations of bortezomib and [<sup>131</sup>I]MIP-1145, synergy was not evident (CI > 1).

### 3.5. Topotecan or AG014699 Enhanced the Delay of the Growth of Melanotic Spheroids Induced by [<sup>131</sup>I]MIP-1145

Multicellular spheroids were treated simultaneously with radiosensitizer and [<sup>131</sup>I]MIP-1145 as this administration schedule was observed to be the most effective means of sterilising clonogens derived from cellular monolayers. [<sup>131</sup>I]MIP-1145 at 3.7 MBq/ml was used in combination treatments because this radioactivity concentration of the single agent induced 53% growth delay (according to AUC determinations), rather than sterilization of A2058 spheroids (Figure 3). The selected concentrations of drugs employed in combination treatments were based upon those dosages, as single agents, that resulted in approximately 50% inhibition of growth of A2058 spheroids, according to AUC determinations. Therefore, topotecan was used at 1M, AG014699 at 10 M and bortezomib at 5 nM. The effects of radiosensitizers and [<sup>131</sup>I]MIP-1145, alone and in combination, on the growth of spheroids derived from A2058 or SK-N-BE(2c) cells are shown in Figures 4 and 5, respectively. Indices of growth delay induced by experimental therapy were the time taken to increase spheroid volume 10-fold (10) and the area under the volume-time curve (AUC). These values are presented in Tables 3 and 4.

In melanotic spheroids, 3.7 MBq/ml [<sup>131</sup>I]MIP-1145 caused a delay in the growth of spheroids (Figure 4), exemplified by a decrease in AUC from 1695 (untreated control) to 569 ([<sup>131</sup>I]MIP-1145 alone). Topotecan, AG014699 and bortezomib also caused delays as single agents at the concentrations used, with AUC values of 986, 653 and 861, respectively. Combination of [<sup>131</sup>I]MIP-1145 with either topotecan or AG014699 resulted in sterilization of spheroids; this sterilization result manifest as a failure to increase in size throughout the duration of the experiment.

Although bortezomib enhanced the growth delay effect of [<sup>131</sup>I]MIP-1145 (AUC of combination was 341), it was not as effective as topotecan or AG014699.

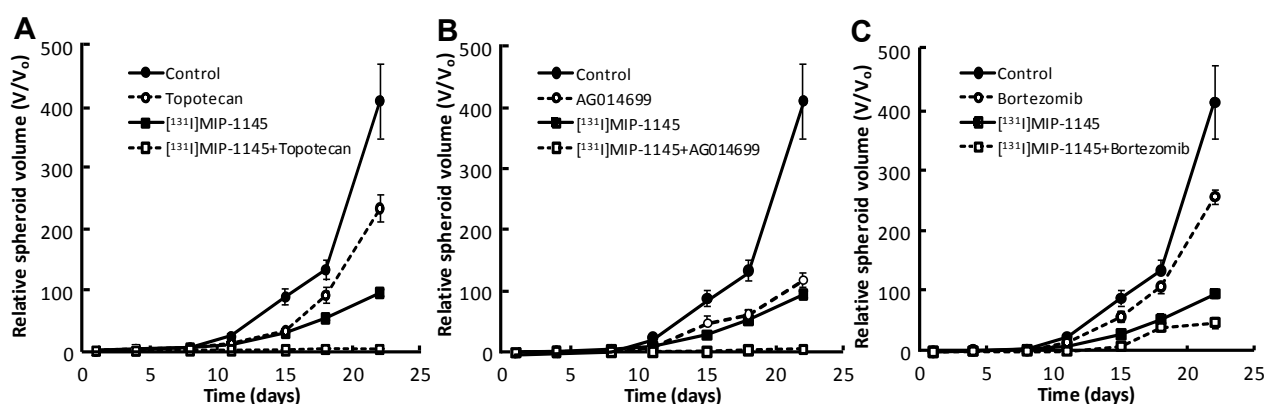
As shown by the concentration response in amelanotic spheroids (Figure 3B), [<sup>131</sup>I]MIP-1145 does not have any effect on spheroid growth. In amelanotic spheroids (Figure 5), treatment with drugs alone significantly delayed spheroid growth as measured by  $\tau_{10}$  and AUC (Table 4). However, the addition of [<sup>131</sup>I]MIP-1145 did not significantly enhance this delay in combination with any of the drugs used.

**Table 3.** Comparison of the effect of single agent treatment with combination treatment on the growth of A2058 spheroids. Data are means  $\pm$  SEM of 24 spheroids per treatment. Kruskal-Wallis and Mann-Whitney tests were performed using SPSS software. \* pertains to drug compared with [<sup>131</sup>I]MIP-1145 plus drug. † pertains to [<sup>131</sup>I]MIP-1145 compared with [<sup>131</sup>I]MIP-1145 plus drug; \*/†  $p < 0.05$ , \*\*/††  $p < 0.01$ .

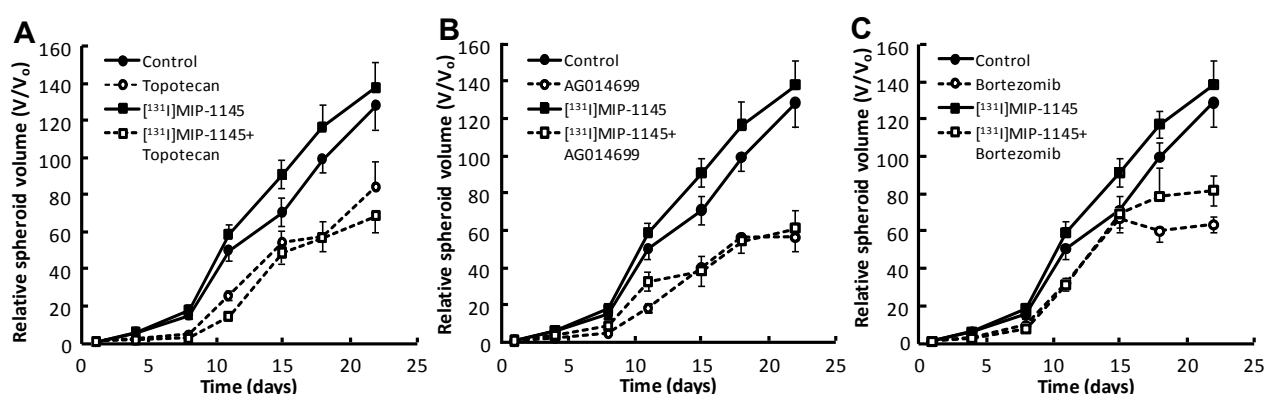
	$\tau_{10}$	AUC
Control	7.8 $\pm$ 1.7	1695 $\pm$ 232
[ <sup>131</sup> I]MIP-1145	9.9 $\pm$ 2.1	569 $\pm$ 38
Topotecan	9.0 $\pm$ 2.1	986 $\pm$ 119
[ <sup>131</sup> I]MIP-1145 + Topotecan	N/A **††	50 $\pm$ 11 **††
AG014699	9.9 $\pm$ 2.2	653 $\pm$ 136
[ <sup>131</sup> I]MIP-1145 + AG014699	N/A **††	52 $\pm$ 14 **††
Bortezomib	8.7 $\pm$ 1.9	861 $\pm$ 149
[ <sup>131</sup> I]MIP-1145 + Bortezomib	14.1 $\pm$ 1.5 *†	341 $\pm$ 91 *†

**Table 4.** Comparison of the effect of single agent treatment with combination treatment on the growth of SK-N-BE(2c) spheroids. Data are means  $\pm$  SEM of 24 spheroids per treatment. Kruskal-Wallis and Mann-Whitney tests were performed using SPSS software. ††  $p < 0.01$  compared to [<sup>131</sup>I]MIP-1145 alone.

	$\tau_{10}$	AUC
Control	5.4 $\pm$ 1.1	1027 $\pm$ 143
[ <sup>131</sup> I]MIP-1145	4.8 $\pm$ 1.1	1319 $\pm$ 105
Topotecan	7.9 $\pm$ 1.0	674 $\pm$ 91
[ <sup>131</sup> I]MIP-1145 + Topotecan	9.1 $\pm$ 1.3 ††	574 $\pm$ 80 ††
AG014699	8.1 $\pm$ 1.1	542 $\pm$ 52
[ <sup>131</sup> I]MIP-1145 + AG014699	7.1 $\pm$ 1.2 ††	610 $\pm$ 93 ††
Bortezomib	7.1 $\pm$ 1.4	725 $\pm$ 67
[ <sup>131</sup> I]MIP-1145 + Bortezomib	7.5 $\pm$ 1.0 ††	824 $\pm$ 101 ††



**Figure 4.** The effect on A2058 spheroid growth of single agent or combination treatment. Multi-cellular spheroids derived from A2058 cells were treated with 3.7 MBq/ml [ $^{131}\text{I}$ ]MIP-1145 and (A) 1  $\mu\text{M}$  topotecan, (B) 10  $\mu\text{M}$  AG014699, (C) 5 nM bortezomib as single agents or in combination. Data are expressed as mean spheroid volume at each time-point divided by original volume ( $V/V_0$ )  $\pm$  SEM of 24 spheroids per treatment.



**Figure 5.** The effect on SK-N-BE(2c) spheroid growth of single agent or combination treatment. Multi-cellular spheroids derived from SK-N-BE(2c) cells were treated with 3.7 MBq/ml [ $^{131}\text{I}$ ]MIP-1145 and (A) 1  $\mu\text{M}$  topotecan, (B) 10  $\mu\text{M}$  AG014699, (C) 5 nM bortezomib as single agents or in combination. Data are expressed as mean spheroid volume at each time-point divided by original volume ( $V/V_0$ )  $\pm$  SEM of 24 spheroids per treatment.

## 4. Discussion

The combination of radiotherapy and chemotherapy is an appealing approach that has led to improved treatment results in many patients with advanced solid tumors [29]. However, response rates for metastatic melanoma using currently available treatments are poor [1]. In this study [ $^{131}\text{I}$ ]MIP-1145, a benzamide compound that has been shown to specifically target melanin [5], was used in combination with potential radiosensitizers in an attempt to maximize damage in malignant deposits.

Our findings indicate that [ $^{131}\text{I}$ ]MIP-1145 was taken up by the melanin-expressing cell line, and this is in line with the previous report of melanin-specific uptake of this agent *in vitro* [5]. The combination index method, based on the median-effect principle of Chou and Talalay [27], was used to analyse synergistic cytotoxicity. Three different administration regimes were evaluated because previous studies of combination treatment comprising radiosensitizers and targeted radionuclides showed that scheduling was an important determinant of synergy [24,25]. Experimental treatment of melanotic cells using topotecan in combination with [ $^{131}\text{I}$ ]MIP-1145 was synergistic

regardless of schedule of administration. This observation is in agreement with reports of topotecan's capacity to radiosensitize a variety of solid tumors [21-23]. The enhancement of efficacy of [ $^{131}\text{I}$ ]MIP-1145 by combining with topotecan is likely the result of inhibition of DNA damage repair, as was previously demonstrated in similar studies assessing the combination treatment of neuroblastoma cells with [ $^{131}\text{I}$ ]MIBG and topotecan [24]. Encouraging results have been obtained using topotecan combined with radiotherapy for patients with lung cancer [26,30]. Neither single agent treatment with [ $^{131}\text{I}$ ]MIP-1145 nor combination treatment with [ $^{131}\text{I}$ ]MIP-1145 and topotecan was effective in the sterilization of amelanotic cells, confirming the melanin specificity of the radiopharmaceutical.

DNA repair is a potential target pathway for tumors and inhibitors of repair have shown to be effective radiosensitizers [14]. In this study, the PARP-1 inhibitor AG014699 showed the greatest enhancing effect when administered either simultaneously or before the addition of [ $^{131}\text{I}$ ]MIP-1145 in melanotic cells, suggesting that scheduling is important with this drug. PARP-1 binds to both single- and double-stranded DNA breaks, partly via the

base excision pathway, which plays an important role in repairing single-strand breaks induced by ionizing radiation [31]. We predict that PARP-1 inhibition promotes increased formation of DNA single strand breaks which are subsequently converted to potentially lethal double strand breaks during cell replication. This is possible with simultaneous administration or administration of inhibitor 24 hours prior to [ $^{131}\text{I}$ ]MIP-1145, whereas administration of AG014699 24 hours after [ $^{131}\text{I}$ ]MIP-1145 is likely to allow sufficient DNA repair to occur. Initial clinical trials have indicated that AG014699 is safe and tolerable and combination treatments with temolozolomide have shown encouraging results in melanoma patients [18,19]. AG014699 has also been used as a radiosensitizer in phase I and II clinical trials in head and neck cancers and CNS neoplasm [32,33], indicating its potential in other solid tumors. Cancers bearing mutations in either the BRCA1 or BRCA2 genes were extremely susceptible to PARP inhibitors [34] and AG014699 is currently being assessed in BRCA-deficient ovarian and breast cancer patients [35]. BRCA mutations have also been associated with some melanomas [36], suggesting that PARP inhibitors would be a particularly useful addition to the treatment of this cancer.

Although bortezomib has anti-tumor activity as a single agent in multiple myeloma [37], its use for solid tumors is controversial [38]. It was toxic to melanoma cells *in vitro* as a single agent [39,40], but displayed minimal activity in patients [41] and was associated with dose-limiting toxicities [42]. In combination studies, bortezomib did not enhance the effects of heat shock protein inhibitors in a melanoma cell line [43] or paclitaxel in melanoma patients [41]. The sequence of administration has been shown to be important when combining bortezomib with chemotherapeutic agents, although conflicting views on the optimal dosing schedule have been reported [44,45]. The radiosensitizing effect of bortezomib has previously been reported [8,46], but was lost when radiotherapy dose was fractionated [47]. In this study, the administration of bortezomib simultaneously with [ $^{131}\text{I}$ ]MIP-1145 in melanotic cells showed no synergistic effect, and less synergy than either topotecan or AG014699 when administered before or after [ $^{131}\text{I}$ ]MIP-1145.

One of the major pathways of bortezomib action is the generation of reactive oxygen species (ROS) [48]. Due to the production of high levels of oxygen-containing radicals during melanin biosynthesis, melanoma cells contain high levels of ROS [49]. Drug or radiation treatment likely results in elevation of ROS levels above a threshold level, causing cell death. It is therefore possible that the radiation- or bortezomib-induced ROS generation is maximal after single agent treatment of the melanoma cells used here, resulting in the lack of additive or synergistic effect of the combination observed in this study. Neither topotecan nor AG014699 are thought to act through ROS generation.

We have previously evaluated the efficacy of targeted radionuclide treatment *in vitro* in a three dimensional tumor spheroid model [50,51]. These models are useful for determining the dependence of novel targeted radionuclide

therapy strategies on cycling and oxygenation status (factors which influence the toxicity of radionuclides) because they are composed of cells in the range of proliferative states. The major target of radionuclide therapy is disseminated malignant deposits of millimetre dimensions. Melanotic and amelanotic spheroids were exposed to a range of doses of [ $^{131}\text{I}$ ]MIP-1145 to determine their responsiveness to the radiopharmaceutical. As expected, melanotic spheroids responded in a concentration-dependent manner, whereas amelanotic spheroids did not show any growth delay in response to treatment. In agreement with the monolayer experiments described above, both topotecan and AG014699 were shown to enhance growth delay in melanotic spheroids, with significant enhancement of [ $^{131}\text{I}$ ]MIP-1145-induced delay in growth when administered in combination. Combined treatment of [ $^{131}\text{I}$ ]MIP-1145 with bortezomib in melanotic spheroids did not result in a significantly enhanced growth delay. As predicted, [ $^{131}\text{I}$ ]MIP-1145 did not have any effect on amelanotic spheroids and therefore did not cause a growth delay when combined with any of the drugs.

The search for a radiopharmaceutical with a selective affinity for melanoma tissue is of utmost importance both for the early detection of local recurrences to allow efficient surgical excision and for treatment of in-transit or developed metastases. Most melanomas are pigmented by the presence of melanin, an intracellular melanocyte pigment. Some melanomas are called amelanotic because they are not black or darkly pigmented. However, even amelanotic melanomas contain some melanin [52,53] which makes this pigment a convenient target for development of radionuclide therapy of metastatic melanoma. This makes [ $^{131}\text{I}$ ]MIP-1145, which specifically targets melanin, an ideal candidate for targeted therapy of melanoma. The feasibility of targeting melanin to deliver cytotoxic radiation to human melanoma cells *in vivo* was demonstrated using a fungal melanin-binding monoclonal antibody with promising therapeutic results [54]. Although targeted therapy was previously thought to be suited to micrometastasis, targeted alpha therapy has been used in a phase I trial for metastatic melanoma [55], giving hope that these therapies can be used for solid tumors. Targeted radionuclide therapy concentrates the effects on tumor cells, thereby increasing the efficacy and decreasing the morbidity of radiotherapy, and there has also been a recent report of a radiolabeled peptide therapy in trial for metastatic melanoma [56], suggesting promise for targeted therapy in this disease. It is now hoped that combination with suitable radiosensitizing drugs will enhance the effect of these targeted radiotherapies.

## 5. Conclusions

The results described in this study suggest that combination of [ $^{131}\text{I}$ ]MIP-1145 with some, but not all, chemotherapeutic drugs could be an effective treatment for melanoma. The specificity of [ $^{131}\text{I}$ ]MIP-1145 to target only melanin-expressing cells means reduced off-target effects,



and in combination with radiosensitizers such as topotecan or AG014699, suggests a promising therapeutic approach. However this will require to be confirmed in other melanin-expressing cells and pre-clinical models.

## Funding

This work was supported by grants from the Scottish Governmental Chief Scientist Office and Molecular Insight Pharmaceuticals Inc.

## Acknowledgements

We thank Dr Sally Pimlott for radiopharmaceutical synthesis.

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